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Veterinary Microbiology 106 (2005) 7–16
veterinary microbiology
www.elsevier.com/locate/vetmic

Genomic sequences of low-virulence avian paramyxovirus-1 (Newcastle disease virus) isolates obtained from live-bird markets in North America not related to commonly utilized commercial vaccine strains

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Received 21 June 2004; received in revised form 1 November 2004; accepted 2 November 2004

Abstract

Avian paramyxovirus 1 (APMV-1), also referred to as Newcastle disease virus (NDV), variants of low virulence were isolated from chickens, ducks and other unidentified species found in live-bird markets of the northeastern United States. These isolates were characterized as APMV-1 by the hemagglutination-inhibition (HI) assay utilizing NDV-specific polyclonal antisera. However, the isolates failed to react with a monoclonal antibody that has specificity for a wide variety of APMV-1 isolates. Although only highly virulent isolates require reporting to international regulatory agencies, the ability to correctly identify APMV-1 types is important for control and regulatory purposes. Protein gel patterns of the purified isolates resembled previously reported APMV-1 and anti-NDV polyclonal sera recognized the viral proteins. For three isolates oligonucleotide primers specific for the nucleoprotein, fusion protein and polymerase genes of NDV were utilized to synthesize cDNA using viral RNA as a template. Approximately 12 kb of the genome was subsequently sequenced for the three isolates that included the nucleoprotein, phosphoprotein, matrix protein, fusion (F) protein, hemagglutinin-neuraminidase protein genes and a 5′ portion of the polymerase gene. The isolates had an F protein cleavage site sequence of ERQER/LVG indicating low-virulence viruses that phylogenetically separated with other unique NDV isolates designated as a lineage 6 genotype. Additionally, a four amino acid insert was detected in the predicted phosphoprotein which complies with the ''rule of six'' among paramyxoviruses. These APMV-1 genotypes have not been previously reported in North

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0378-1135/$ – see front matter
doi:10.1016/j.vetmic.2004.11.013

America and further substantiate the heterogeneous genetic nature of these commercially important pathogens found worldwide.

Keywords: Veterinary medicine; Emerging diseases; Molecular epidemiology; Mononegavirales

1. Introduction

Newcastle disease virus (NDV) is one of the most serious infectious diseases of poultry. The virus belongs to the *Avulavirus* genus within the family Paramyxoviridae, subfamily Paramyxovirinae, in the order Mononegavirales and is designated avian paramyxovirus-1 (APMV-1). Nine avian paramyxovirus serotypes (APMV-1 to APMV-9), of which APMV-1 is the most economically important, have been identified among these virus types (Alexander, 2003). The enveloped virus has a negative-sense, single-stranded RNA genome which codes for six proteins including a nucleoprotein (N), phosphoprotein (P), matrix (M) protein, fusion (F) protein, hemagglutinin-neuraminidase (HN) protein and an RNA directed RNA polymerase (L) in the 3′ to 5′ direction (de Leeuw and Peeters, 1999). The genomes of several rubulaviruses, wherein NDV was formerly categorized, contain a small hydrophobic (SH) protein gene that is also not present among the respiroviruses (Lamb and Kolakofsky, 2001). Interestingly, the avian paramyxovirus-6 (APMV-6) genome was reported to contain a putative SH protein gene (Chang et al., 2001). Transcription occurs with the N:RNA complex associated with P and L proteins during viral replication. These proteins associate with the cytoskeleton shortly after synthesis and N:RNA, P and L are sufficient for transcriptional activity in cell-free extracts in vitro (Hamaguchi et al., 1983). The M proteins of paramyxoviruses are relatively conserved and structurally form a complex between the nucleocapsid with the surface glycoproteins (Rima, 1989). There are two surface glycoproteins of the virion and the F protein is a type I integral membrane protein with the transmembrane domain located in the carboxyl-terminal region followed by a short cytoplasmic domain. It is synthesized along the endoplasmic reticulum as an inactive F_0 precursor that must be cleaved to the F_2 and F_1 for fusion activity (Morrison, 2003). The HN glycoprotein of NDV has the transmembrane region located in the amino-terminal region making it a type II integral membrane protein that is involved with viral attachment to cells via sialic acid receptors. The HN protein promotes fusion by F1 and has both hemagglutination properties as well as a neuraminidase activity (Yusoff and Tan, 2001).

Isolates of NDV have been historically categorized into three pathotypes depending on the severity of disease caused by an isolate (Alexander, 2003). Lentogenic NDV isolates do not usually cause disease in adult birds, are considered of low virulence and are utilized extensively as live-virus vaccines. Viruses of intermediate virulence that cause respiratory disease, but are not usually fatal, are termed mesogenic (Hanson and Brandly, 1955). Among the highly virulent velogenic NDV isolates, the viscerotropic and neurotropic forms are reported worldwide (Alexander, 2003). The primary molecular determinant for NDV virulence is the F protein cleavage site amino acid sequence (Nagai et al., 1976; Glickman et al., 1988) and the ability of specific cellular proteases to cleave the F protein of different pathotypes (Gotoh et al., 1992; Ogasawara et al., 1992). Dibasic amino acids surrounding glutamine at position 114 are present in the F protein cleavage site of mesogenic or velogenic strains, while the F protein of lentogenic NDV isolates lack this motif (Nagai et al., 1976; Glickman et al., 1988). Presence of dibasic amino acids in the F protein sequence allows for systemic spread of velogenic NDV, whereas replication of lentogenic NDV is primarily limited to mucosal surfaces of avian tissues in the respiratory and gastrointestinal systems (Ogasawara et al., 1992). All NDV isolates will replicate in chicken embryo kidney cells (King, 1993), presumably due to the presence of a required protease (Ogasawara et al., 1992). However, lentogenic strains must have proteases added to cultured cells for replication in

avian fibroblasts or mammalian cell types, whereas mesogenic and velogenic NDV do not have this requirement (King, 1993; Nagai et al., 1976).

Outbreaks of Newcastle disease among poultry occur worldwide and highly virulent forms are categorized as List A diseases that must be reported to the Office International des Epizooties (OIE) which result in severe trade limitations (Alexander, 2003). Weekly and bimonthly reports revealed no change in the global scale of outbreaks since 1997 (http://www.oie.int). It is important to note that APMV-1 infects approximately 236 species of pet and free living birds in addition to domestic avian species including pigeons because they may transmit the virus (Kaleta and Baldauf, 1988). Recently there has been increased concern with transmission of disease agents among live-bird markets in the U.S. since these establishments often contain multiple species housed in close quarters. Both avian influenza (Bulaga et al., 2003) and NDV (King and Seal, 1997; Kommers et al., 2003) have been isolated from birds in these markets. In the case of NDV, some of the strains have not been amenable to molecular typing methods and herein we report the genomic sequence for three of these isolates.

2. Materials and methods

2.1. APMV-1 isolates, histories and biological characterizations

Several low-virulence APMV-1 isolates were obtained by the National Veterinary Services Laboratories (NVSL), Animal, Plant Health Inspection Services (APHIS), USDA during surveillance of live-bird markets in the northeastern United States. Subsequently three isolates were examined for biological properties and nucleotide sequence of the genome. Inoculation and determination of the intracerebral pathogenicity index (ICPI) along with hemagglutination-inhibition (HI) assays were conducted on selected viruses as described by the American Association of Avian Pathologists (AAAP) for NDV isolates (Alexander, 1999). Monoclonal antibody (B79) that has specificity for a wide variety of APMV-1 isolates was also utilized for HI assays (Lana et al., 1988).

2.2. Molecular characterizations including RNA extraction, oligonucleotide primers, RT-PCR and cloning of amplification products

Isolates duck/U.S./119535-3/2001, duck/U.S./154979-1/2002 and chicken/U.S./101250-2/2001 were replicated in specific pathogen-free (SPF) embryonated chicken eggs (Alexander, 1999). Subsequently, viruses were purified by velocity gradient centrifugation in sucrose followed by SDS-polyacrylamide gel electrophoresis (Collins et al., 1993). Western blotting and probing with polyclonal anti-NDV chicken sera was completed by established procedures (Gershoni, 1988; Nesbitt and Horton, 1992). Genomic RNA was extracted directly from the allantoic fluid of each isolate using a Trizol (Invitrogen, Carlsbad, CA) RNA extraction kit, according to the manufacturers instructions. Preparation of the viral RNA, reverse transcription, and polymerase chain reaction amplification methods were conducted by use previously described assays (Seal et al., 1995; Lomniczi et al., 1998; Aldous et al., 2003), with the exception that initial amplifications were conducted at 35C annealing temperatures. After obtaining initial sequence ‘‘primer walking’’ was completed utilizing 5′ RACE (Frohman, 1993). All RT-PCR primers amplified are available from the authors upon request. RT-PCR was conducted with Superscript reverse transcriptase (Invitrogen, Carlsbad, CA) and Elongase (Invitrogen, Carlsbad, CA) polymerase (Kotewicz et al., 1988; Lewis et al., 1992). Amplification products were cloned (Mead et al., 1991) with the TA Cloning System (Invitrogen, Carlsbad, CA) when not sequenced directly off the PCR product.

2.3. Nucleotide sequencing and phylogenetic analyses

Amplification products were purified with Microcon (AMICON Bioseparations) spin filters and spectrophotometrically quantified along with sequencing of cloned material. Direct double-stranded nucleotide sequencing was completed with Taq polymerase (Applied Biosystems, Inc., Foster City, CA) with the oligonucleotide primers used for RT-PCR or internally derived primers. Determination of the nucleotide sequences by the dideoxynucleotide

chain termination method was used with fluorescence-labeled dideoxynucleotides and an automated nucleic acid sequencer (Smith et al., 1986). Nucleotide sequence editing, analysis, prediction of amino acid sequences and alignments were conducted with DNA Star™ and IntelliGenetics GeneWorks 2.5.1™ software. The phylogenetic analyses presented were completed with Phylogenetic Analysis Using Parsimony (PAUP) Version 4.0b software utilizing both neighbor joining and parsimony searches (Swofford, 1998) with 2000 bootstrap replicates (Hedges, 1992).

3. Results and discussion

3.1. Virus isolates, virulence assays and immunological analyses

Several paramyxoviruses including duck/U.S./119535-3/2001, duck/U.S./154979-1/2002 and chicken/U.S./101250-2/2001 were isolated from birds in live-bird markets that replicated to high titer in embryonated chicken eggs, but the infected embryos had mean death times (MDT) of 96 h and intracerebral pathogenicity indices (ICPI) of zero. The MDT and ICPI results could be interpreted such that these isolates were of low virulence, however more refined characterizations were necessary to specifically identify isolate type. The isolates were typed as NDV positive by HI utilizing polyclonal anti-NDV sera. However, a monoclonal antibody (B79) that routinely reacts with most all NDV isolates tested to date by NVSL (Lana et al., 1988) was negative by HI. Consequently, the isolates were purified by velocity sedimentation in sucrose gradients for PAGE and western blotting. All the isolates had protein gel patterns that resembled NDV (Collins et al., 1993) and were recognized by polyclonal anti-NDV sera (data not shown).

3.2. Genomic sequence and predicted amino acid alignments

Aliquots of genomic RNA for viruses duck/U.S./119535-3/2001, duck/U.S./154979-1/2002 and chicken/U.S./101250-2/2001 were purified from infective allantoic fluid as well from virions isolated by velocity centrifugation. Following purification of genomic RNA, low stringency annealing of primers was utilized to construct cDNA from the genomes of three isolates. A contiguous sequence of 11,967 nucleotides was obtained that incorporated the N, M, P, F and HN genes along with the 5′ end of the L gene (Krishnamurthy and Samal, 1998; de Leeuw and Peeters, 1999). The conserved consensus transcription start sequence of ACGGGUAGAA was located at the 5′ end of each gene which is transcribed as the 5′ sequence of the mRNA. The three low-virulence isolates from live-bird markets shared 98% nucleotide sequence identity indicating they were closely related with each other. The three live-bird market isolates were then compared to other NDV isolates for which full-length genomic sequences were available. Overall, the identity of the live-bird market isolates RNA with other NDV genomic sequences was approximately 74% indicating a wide divergence of the live-bird market isolates with the standard B1-type vaccine strains (NC_002617) or the highly virulent isolate from the southwestern U.S. obtained during the 2002–2003 outbreak (AY562987).

Alignment of the predicted amino acid sequences for each genomic open reading frame (ORF) revealed that many of the features common to proteins among paramyxoviruses were retained by the live-bird market isolates. There was no predicted SH gene as in the case of other APMV-1 isolates (de Leeuw and Peeters, 1999), contrary to what was reported for APMV6 (Chang et al., 2001). The predicted N protein contained all the amino acids highly conserved among paramyxoviruses including the strictly conserved SFAMG motif (Morgan et al., 1984) with a hypervariable portion in the C-terminus as previously identified (Seal et al., 2002). Although the predicted N protein contained the exact number of amino acids as other APMV-1 isolates, the predicted P protein had a four amino acid insert in the sequence among the three live-bird isolates resulting in a predicted protein of 399 amino acids rather than 395 (Fig. 1). This four amino acid insert is the result of an insertion of 12 nucleotides that is unique to these isolates and still allows for adherence to the ‘‘rule of six’’ common among paramyxoviruses (Kolakofsky et al., 1998). Also, identified were the presence of other start sites in the P gene (including position 441) along with the highly conserved CTAAAAAGGGCCCAC transcriptional editing site detected among genomes of other

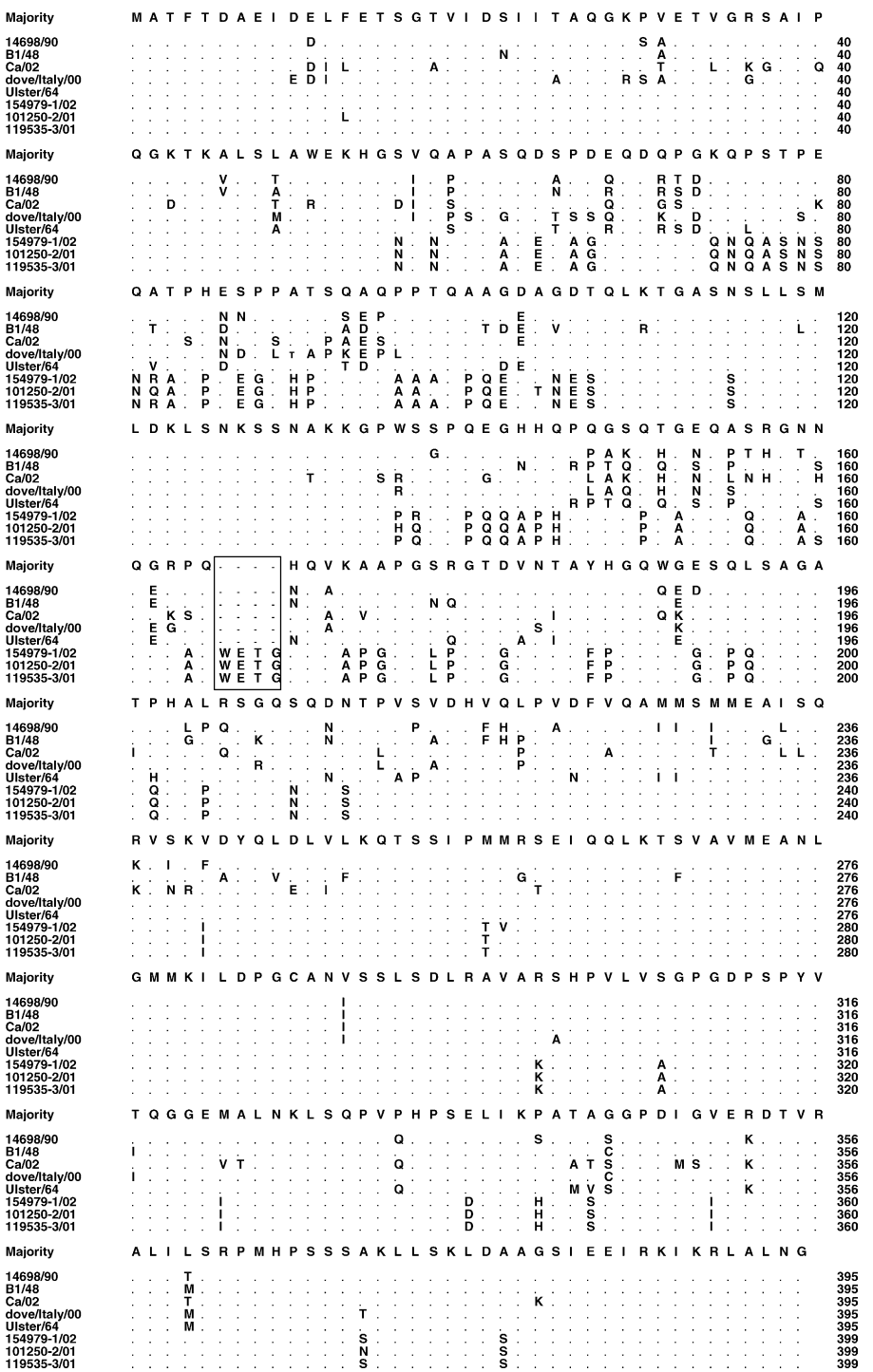

Fig. 1. Alignment of the predicted phosphoprotein amino acid sequence demonstrating presence of the four residue insertion among low-virulence, live-bird market APMV-1 isolates from the U.S. The three live-bird market isolates' predicted amino acid phosphoprotein sequences were aligned with those from representative Newcastle disease virus isolates and the insert was boxed.

NDV isolates (Steward et al., 1993; Locke et al., 2000).

Along with N and P the replication complex of paramyxovirses is composed of the L polymerase protein (Hamaguchi et al., 1983) that contains six principal domains homologous among negative strand RNA-virus polymerases (Poch et al., 1990). Overall, the predicted L protein was highly conserved among the live-bird isolates including the QGDNQ active site from positions 749 to 753 as previously reported among other NDV strains (Wise et al., 2004). However, the live-bird market isolates had several ''signature'' sequences such as a VRLDI for consensus ASIEV from position 99 to 103, an HKIR for consensus NNVP from position 146 to 149, a QD for KE at 498 to 499, a TK for SR at positions 701 to 702, an SG for PD 892 to 893, an SRK for TRR from positions 1069 to 1071 and an A for S at position 1102 in the N-terminal half of the protein. The other highly conserved protein, M, for the live-bird market isolates was predicted to contain 364 amino acids as reported previously for other NDV strains (Seal et al., 2000) with several conserved substitutions such as an S for N at position 70, a TS for A/VT at residues 73–74, an A for T and I for V at positions 112 and 115, an EN at for DS at residues 228–229 and an R for K and L for I at positions 250 and 261 in the predicted M amino acid sequence.

The interactive surface glycoproteins of paramyxoviruses include the F and HN of NDV (Morrison, 2003; Yusoff and Tan, 2001). The first approximately 30 amino acids of the predicted F2 cleavage product were the most highly variable among the proteins (Toyoda et al., 1989; Seal, 2004) and this was clearly evident among the live-bird market isolates. This included the lack of a C at position 25, but did include the C at residue 75 required for linkage with F1 following cleavage activation (Chen et al., 2001a,b; McGinnes et al., 2001). The F cleavage activation site was ERQER/LVG lacking dibasic amino acids at positions 112 and 115 along with an L rather than F at residue 117 indicating these live-bird market isolates were low-virulence isolates by OIE standards (Alexander, 2003). The glycosylation sites present among other APMV-1 isolates were conserved in the live-bird market F proteins as were the sites for disulphide bond formation. The fusion peptide sequence in F was highly conserved among NDV isolates. However, the transmembrane (TM) region contained numerous conserved substitutions. This included a non-conserved C for F at residue 514 of the predicted F protein TM region also detected in the NDV Ulster strain (Seal, 2004).

The predicted HN lengths were 572, 574 and 581 amino acids for the three live-bird market isolates depending on location of the stop codon (Sakaguchi et al., 1989) with no differences in the overall length of the gene. The HN receptor binding site involves positions E401, R416 and Y526, while NA activity was determined to be at residues R174, R416 and R498 (Connaris et al., 2002; Ioroi et al., 2001) which conforms to the hypothesis that receptor recognition as well as NA activity occur as a single site dependent upon HN protein conformation (Crennell et al., 2000; Connaris et al., 2002). All these positions were conserved among the live-bird market isolates. Several substitutions were detected in the TM region (Millar et al., 1986) of the predicted HN protein including T for A at residue 28 and an F for Y at position 46 common among the live-bird market isolates. All the conserved cysteines were present in the predicted HN amino acid sequence required for disulphide bond formation (Pitt et al., 2000). However, two predicted glycosylation sites were absent due to an A for N substitution at position 120 and a G for N at residue 538. Virus neutralization sites at residues 494 and 569 (Iorio et al., 1991) were highly variable among NDV isolates and numerous other substitutions were identified throughout the predicted HN protein (Seal, 2004), many of which could account for the inability of monoclonal antibody B79 to react with the live-bird market isolates.

3.3. Phylogenetic analyses of live-bird market NDV isolates from the U.S.

Several representative isolates were compared based on a portion of the genome encoding the F cleavage activation site (Fig. 2) for which genotypes have been designated and representative isolates compared for epidemiological investigations (Lomniczi et al., 1998; Aldous et al., 2003). Duplicate results were obtained utilizing either distance or parsimony methods. These analyses resulted in the low-virulence, live-bird market isolates segregating with what was designated genotype 6 and monoclonal

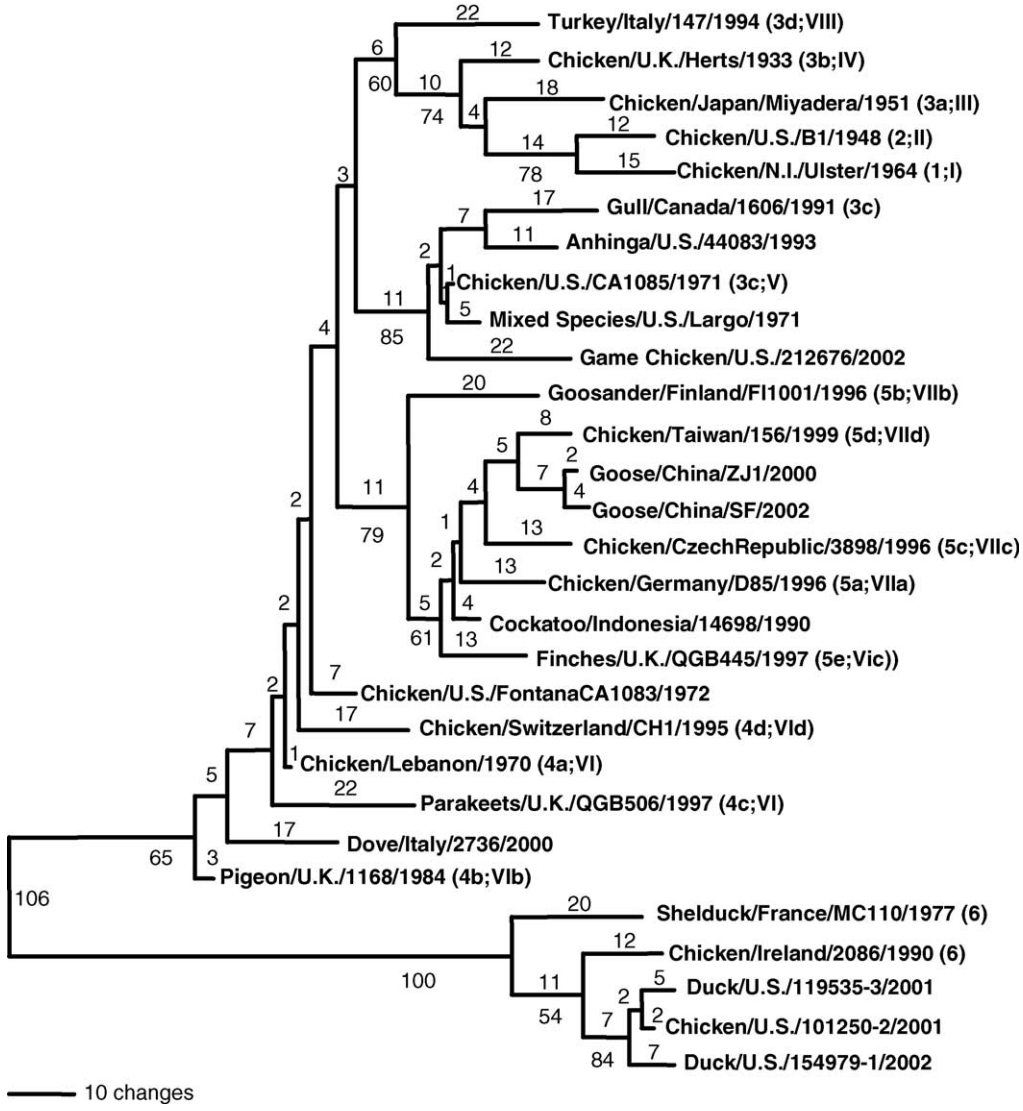

Fig. 2. Phylogenetic analysis based on fusion protein cleavage site sequences from APMV-1 isolates representing all currently designated genotypes. Following alignment of nucleotide sequences surrounding the fusion protein cleavage site phylogenetic relationships were determined by parsimony analysis. Numbers above the lines represent absolute distances and those below represent bootstrap confidence levels.

antibody group H (Aldous et al., 2003) viruses. Following phylogenetic analysis based on the F gene sequences a comparison was completed utilizing the full-length genomic sequences available for NDV isolates. The phylogenetic analyses again resulted in the live-bird market APMV-1 isolates segregating as an outgroup relative to all other isolates (Fig. 3). These data also corroborated the genotype designations reported by other investigators (Lomniczi et al., 1998; Aldous et al., 2003), but whether or not true genomic subtypes occur naturally remains to be further substantiated by analyses of more full-length APMV-1 genome sequences. Although the live-bird market NDV isolates examined from the U.S. were all low-virulence, it is important to note that there was one virulent isolate among the genotype 6 lineage of viruses (Aldous et al., 2003). It was speculated that this virulent virus may have arisen by mutation

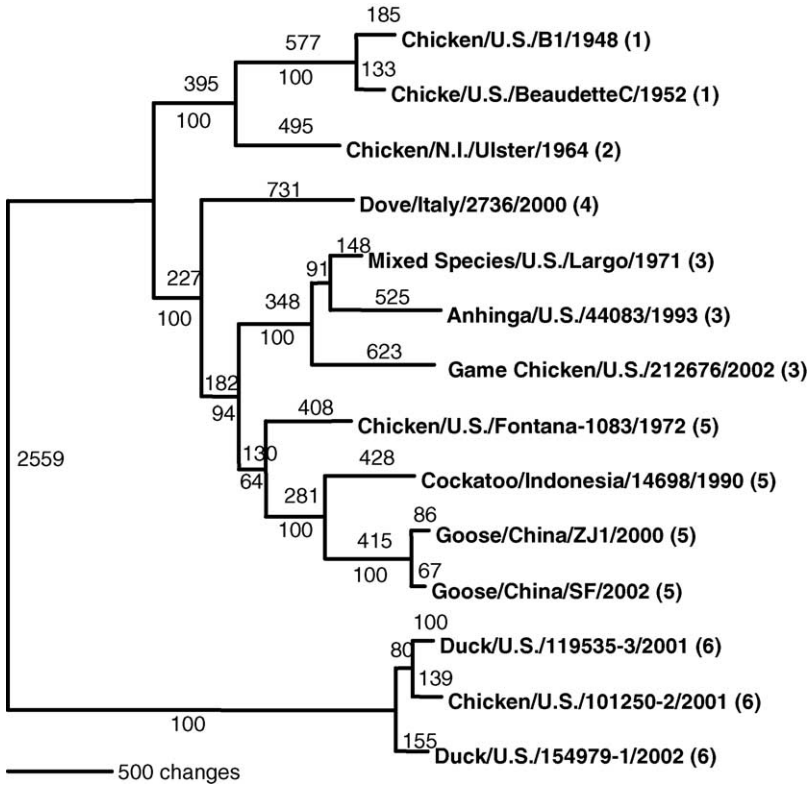

Fig. 3. Phylogenetic analysis based on near full-length genomic sequences of the U.S. live-bird market APMV-1 isolates. Following alignment of those APMV-1 genome sequences available, phylogenetic relationships were determined by parsimony analysis with mid-point rooting. Numbers above the lines represent absolute distances and those below represent bootstrap confidence levels.

(Collins et al., 1998) and fortunately despite the variation commonly utilized vaccines did protect against challenge (Alexander et al., 1992). This situation along with the evolution of low to high virulence NDV during an outbreak in Australia (Gould et al., 2001) further confirm the importance of continued monitoring of APMV-1 isolates for disease control.

Acknowledgments

We thank Robin Kuntz for assistance in cloning APMV-1 genes and Philip Curry for technical assistance. Joyce Bennett performed the automated nucleotide sequencing. These studies were supported by ARS, USDA CRIS project number 6612-32000-015-00D-08, Homeland Security Funds 346-6612-480 (M.W.) and by the Animal and Plant Health Inspection Service of USDA. Nucleotide sequences reported herein have accession numbers are AY626266 for isolate 119535, AY626267 for isolate 154979 and AY626268 for isolate 101250.

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